

The dynamics of HCV-specific antibody responses in HIV/HCV patients on long-term antiretroviral therapy

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Abstract

Antibody responses have not been fully characterised in chronically HIV/HCV patients receiving antiretroviral therapy (ART). Seventeen HIV/HCV patients receiving ART were followed for a median (range) interval of 597 (186-766) weeks. Prior to ART, HIV/HCV patients had lower levels of antibodies reactive with HCV core and JFH-1, and lower genotype cross-reactive neutralising antibodies (nAb) titres than HCV patients. Levels of JFH-1 reactive antibody increased on ART, irrespective of CD4⁺ T-cell counts or changes in serum ALT levels. The appearance of nAb coincided with control of HCV viral replication in five HIV/HCV patients. In other patients, HCV viral loads remained elevated despite nAb responses. Sustained virological responses following HCV therapy were associated with reduced antibody responses to JFH-1 and core but elevated responses to non-structural proteins. We conclude that nAb responses alone may fail to clear HCV, but contribute to control of viral replication in some HIV/HCV patients responding to ART.

Key words: antiretroviral therapy, HCV, HIV, neutralising antibodies

1. Introduction

Co-infection with hepatitis C virus (HCV) is common in human immunodeficiency virus (HIV)-infected patients as the viruses share routes of transmission. Co-infected patients have higher HCV loads and faster progression to liver disease. Antiretroviral therapy (ART) has improved life expectancy, so more cases of HCV-associated liver disease arise. Beneficial effects of ART may not be as great in co-infected patients [1, 2], but HCV-specific cell-mediated immune responses are restored [3, 4]. In some patients, this coincided with elevated serum alanine aminotransferase (ALT) levels [5-8] in a phenomenon known as immune restoration disease (IRD) [6, 8]. Longitudinal studies of antibody responses in HIV/HCV patients receiving ART are limited. In a three year study, HIV/HCV patients were unable to normalise circulating B-cell counts or serum total IgG levels [9]. Titres of HCV-reactive antibodies increased after 1 year of ART, with no changes in HCV [viral](#) loads [10].

The establishment of a cell-culture system for HCV has allowed evaluation of antibodies that can neutralise HCV infection. Neutralising antibody (nAb) can be detected using the HCV genotype 2a strain, JFH-1, in HCV mono-infected patients chronically infected with [different](#) HCV genotype [11, 12]. HIV co-infection reduced nAb levels in a cross-sectional study [13], but we have found no longitudinal data examining HIV/HCV patients [after](#) starting ART. We present an extended analysis of HCV antibody responses detected using JFH-1, and recombinant core and non-structural antigens. Changes observed on ART are analysed in individual patients to dissect out causes and consequences of perturbations in the HCV viral loads and CD4⁺ T-cell counts.

2. Materials and Methods

2.1 Patients and controls

HIV/HCV patients (n=17) attending Outpatient Clinics at Royal Perth Hospital (Western Australia) were monitored for a median (range) interval of 597 (186-766) weeks after starting ART (triple therapy with a protease inhibitor or non-nucleoside reverse transcriptase inhibitor). Some patients had participated in previous studies [6, 8]. Initial diagnoses were based on serology and confirmed by COBAS AMPLICOR™ HCV Test (Roche Diagnostics). Four patients also received anti-HCV therapy [during the follow-up period](#). We included seventeen HCV mono-infected patients attending Royal Perth Hospital, matched for sex and HCV genotype to co-infected patients. HCV mono-infected patients were negative for hepatitis B surface antigens and antibodies to HIV, and had not been treated with IFN α /ribavirin. Seventeen healthy individuals with no reported exposure to HCV or HIV were included as controls. All subjects gave their informed consent and the study was approved by the Human Ethics Committee of Royal Perth Hospital.

2.2 Biochemical markers, CD4⁺ and CD8⁺ T-cell counts and HIV RNA levels

Serum ALT levels were measured on an automated analyser (Hitachi 917: Roche Diagnostics). CD4⁺ and CD8⁺ T-cell counts were determined from fresh blood specimens by standard flow cytometric analyses. Plasma HIV RNA levels were determined by RT-PCR (Amplicor™, Roche Diagnostics). Lower limits of the assay were 2.6 log₁₀ copies/mL from 1996 to 1998 and 1.7 log₁₀ copies/mL from 1998.

2.3 HCV RNA levels and HCV genotyping assay

RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). HCV RNA levels were quantitated using specific primers and Taqman® probes for the conserved 5'UTR region [14] and PCR conditions described previously [15]. For HIV/HCV patients, HCV

genotype was determined by RT-PCR with a melting curve analysis using FRET probes [16]. HCV mono-infected patients were genotyped using LiPA Kits (Inno-Lipa, Innogenetics, Ghent, Belgium).

2.4 Total IgG and CMV antibody ELISAs

Plates were coated overnight with 5µg/mL polyvalent goat antihuman immunoglobulin (Invitrogen), washed with 0.05% Tween/PBS and blocked with 5% BSA/PBS. Plates were washed again and plasma/serum serially diluted in 2% BSA/PBS was added. Human IgG (0.5µg/mL, Sigma, Castle Hill, NSW, Australia) was diluted in 2% BSA/PBS as a standard. Bound Ig was detected with horseradish peroxidase conjugated anti-human IgG (Jackson ImmunoResearch, West Grove, PA), followed by tetramethylbenzidine substrate (Sigma). Reactions were stopped with 1M H₂SO₄ and absorbance measured at 450nm. The coefficient of variance for the assay was 11%.

To assess CMV-reactive antibody, plates were coated with a lysate of human foreskin fibroblasts infected with CMV AD169 as previously described [17]. The coefficient of variance was 13%.

2.5 HCV antibody ELISAs

Production of HCV (JFH-1) in human hepatoma HuH-7 cells has been previously described [18]. JFH-1 infected cells were resuspended in buffer containing protease inhibitors and subjected to decompression lysis. Lysates from the same number of uninfected HuH-7 cells were prepared as a negative control. Microtitre plates were coated with HuH-7 lysates, or 0.5µg/mL recombinant HCV core (genotype 1b), NS3 (genotype 1b), NS4 (genotype 1a) or NS5 antigens (MyBioSource, San Diego, CA). Plasma from an individual positive using AxSYM HCV

Version 3.0 (Abbott, Chicago, IL) was assigned an arbitrary unit value as a standard. JFH-1 antibody levels were determined by subtracting the value obtained with uninfected cells. Coefficients of variance were: 13% (JFH-1), 11% (HCV core), 6.3% (HCV NS3), 6.0% (HCV NS4) and 5.3% (HCV NS5).

2.6 HCV Neutralisation Assay

Viral supernatants harvested from JFH-1-infected cells were stored at -80°C. Viral titer was ascertained by titrating supernatants and incubating on HuH-7 cells in a 96-well plate and counting the foci from duplicate wells to yield foci forming units/mL (FFU/mL). HCV neutralisation assay have been previously described [18]. HCV foci were counted using an AID ELISpot Reader v2.9 software (Autoimmun Diagnostika GmbH, Strassberg, Germany). The 50% neutralising antibody (nAb₅₀) titre is the plasma dilution required to inhibit JFH-1 infection by 50%.

2.7 Statistics

Data are presented as median (range). Statistical analyses were performed using GraphPad Prism Version 5.01 (San Diego, CA). Differences between study groups were evaluated using the non-parametric Mann-Whitney Tests. Correlations were assessed using Spearman's Rank Correlation Coefficient. *P*-values<0.05 are considered statistically significant.

3. Results

3.1 Immunological, biochemical and virological profiles of patients during ART

HIV/HCV patients (**P1-17**) are described in Table 1. HIV/HCV and HCV patients had similar gender distributions, CD8⁺ T-cell counts and HCV genotype (Supplementary Table 1).

HIV/HCV patients were significantly younger and had lower serum ALT levels and CD4⁺ T-cell counts than HCV mono-infected patients. Seven HIV/HCV patients (**P1, P2, P7, P10, P11, P12, P17**) started ART with CD4⁺ T-cell counts below 50 cells/ μ L.

Statistical analyses spanned 520 weeks, with most patients followed to this timepoint. Median (range) CD4⁺ T-cell counts increased from 150 (14–149) to 400 (200–1274) cells/ μ L at 520 weeks (Supplementary Figure 1a), with a significant increase at 104 weeks ($p=0.05$) and smaller increments thereafter. Eight patients (**P1, P2, P7, P10, P11, P12, P15, P17**) achieved more than a 4-fold increase in CD4⁺ T-cell counts. CD4⁺ T-cell counts remained below 200 cells/ μ L in **P17**.

Median serum ALT levels increased from 41 (15–86) to 81 (29–663) U/mL at week 52 ($p=0.006$) (Supplementary Figure 1b), and were elevated above baseline at 104 ($p=0.07$), 208 ($p=0.01$), 312 ($p=0.06$) and 416 ($p=0.006$) weeks. Ten patients experienced a >3-fold rise in serum ALT to over 100 U/mL on ART (**P1, P3, P4, P6, P9, P10, P11, P13, P16, P17**).

Persistent suppression of HIV replication (defined as less than one rebound in HIV viremia >3.0 log₁₀) on ART was achieved in **P1, P2, P7, P8, P10, P12, P14** and **P15**.

3.2 Low JFH-1 antibody responses did not reflect a global defect in antibody responses

Levels of total IgG were higher in HIV/HCV patients prior to starting ART than HCV mono-infected patients ($p=0.0003$) and controls ($p=0.006$) (Figure 1a). Amongst the individuals who were CMV seropositive (15 HIV/HCV patients, 10 HCV patients and 12 controls), CMV antibody levels were higher in HIV/HCV patients than mono-infected patients ($p=0.01$) and

controls ($p=0.003$) (Figure 1b). CMV antibody levels were similar in mono-infected patients and controls.

As this does not suggest a global defect in antibody responses, it is striking that HIV/HCV patients had lower levels of antibody reactive to JFH-1 and genotype cross-reactive nAb₅₀ titres than HCV mono-infected patients ($p<0.0001$), although levels were higher than in controls ($p<0.0001$) (Figures 1c and 1h). **P1, P5, P6, P9, P11, P12, P15, P16 and P17** had undetectable or low nAb₅₀ titres. HCV core antibody levels were also lower in HIV/HCV patients than HCV mono-infected patients ($p=0.0002$), but higher than controls ($p<0.0001$) (Figure 1d).

In contrast, levels of antibody to NS3 and NS5 antigens were higher in HIV/HCV patients than HCV mono-infected patients ($p<0.0001$) (Figures 1e and 1g). NS4 antibody levels were similar in both patient groups ($p=0.41$) and higher than controls ($p<0.0001$) (Figure 1f).

3.3 Levels of JFH-1 antibody are affected by CD4⁺ T-cell counts but not by HCV genotype

CD4⁺ T-cell counts correlated weakly with JFH-1 reactive antibody levels in co-infected patients prior to starting ART ($r=0.30$, $p=0.08$), but not in HCV mono-infected patients ($r=-0.19$, $p=0.49$). A significant direct relationship was found between levels of antibody detected in the JFH-1 ELISA and nAb₅₀ titres in co-infected patients ($r=0.61$, $p=0.009$), with a weaker correlation in HCV mono-infected patients ($r=0.39$, $p=0.12$). Median (range) levels of JFH-1 reactive antibody for co-infected patients with HCV genotype 1, HCV genotype 2 and HCV genotype 3 were similar [12440 (0–430852), 79952 (9665–150238) and 14896 (472–251112), respectively]. Median (range) nAb₅₀ titres were also unaffected by HCV genotype [genotype 1: 980 (0–7290),

genotype 2: 3700 (400-7000) and genotype 3: 1750 (0-5500); $p=0.80$]. nAb₅₀ titres did not correlate with any immunological or biochemical parameters evaluated in both patient groups.

3.4 Co-infected patients retained elevated levels of total IgG and antibody to CMV on ART

Twelve of the 17 HIV/HCV patients had total IgG levels above mono-infected patients and controls in at least 1 follow-up sample (Figure 2a). For example, **P2** began ART with high levels that normalised after 104 weeks, whilst **P9** started with normal levels which rose and only normalised after 465 weeks. Only **P14** retained low levels over 718 weeks of follow-up. CMV-reactive antibody rose during the first year of ART and remained highest in co-infected patients (Figure 2b). However, **P3** and **P14** were CMV seronegative at baseline and did not seroconvert, and CMV antibody levels declined in **P1**, **P2**, **P10** and **P11**.

3.5 JFH-1 reactive antibody responses increased on ART, irrespective of baseline CD4⁺ T-cell counts or changes in serum ALT levels during therapy

With the exception of **P11**, JFH-1 reactive antibody levels increased in most patients within 52 weeks on ART (Figure 2c) coincident with a rise in genotype cross-reactive nAb₅₀ titres (Figure 2d). For **P1**, **P5** and **P16**, the rise in nAb₅₀ titres was delayed (210, 257 and 91 weeks, respectively). After 205 (186-225) weeks, co-infected patients attained nAb₅₀ titres similar to mono-infected patients, whereas levels of JFH-1 antibody remained lower than in mono-infected patients. HCV core antibody levels were significantly lower than mono-infected patients at baseline and during the first few years of ART, but normalised after 258 (242-270) weeks (Supplementary Figure 2a). HCV NS3 and NS5 antibody levels also began low and normalised after 522 (504-538) weeks (Supplementary Figures 2b & d), whereas NS4 antibody levels were stable on ART and similar to mono-infected patients (Supplementary Figure 2c).

When patients were stratified by CD4⁺ T-cell counts at recruitment, JFH-1 antibody responses were similar at equivalent timepoints (Supplementary Figures 3a & b). Interestingly, patients with <50 CD4⁺ T-cells/ μ L at baseline developed nAb responses more rapidly than patients with >50 CD4⁺ T-cells/ μ L (52 *versus* 260 weeks) (Supplementary Figure 3b). In contrast, JFH-1 reactive antibody levels increased significantly by week 52 in both patient groups (Supplementary Figure 3a). There were no statistical differences in antibody responses between patients stratified by serum ALT levels (data not shown).

3.6 JFH-1 antibody responses reflect HCV viral loads during ART

Longitudinal samples identified three patterns associating JFH-1 antibody responses with HCV viral loads during ART. Informative examples are shown in Figure 3.

The first pattern was elevated HCV viral loads despite the development of antibody responses. **P8** and **P13** (Figure 3a) were followed for 637 and 597 weeks, respectively, with two treatment interruptions triggering a rebound in HIV viremia and a decrease in CD4⁺ T-cell count. JFH-1 antibody levels increased within the first year of ART and declined when ART was interrupted. HCV viral loads increased after weeks 144 and 22 (**P8** and **P13**, respectively) and remained elevated. The rise was accompanied by an increase in nAb₅₀ titres. Similar antibody profiles were seen for patients **P4**, **P5**, **P9**, **P14** and **P16** (data not shown).

The second pattern was an inverse relationship between antibody responses and HCV viral loads which was seen for patients **P1**, **P3**, **P6**, **P7**, **P10**, **P12**, **P15** and **P17**. For example; **P12** (Figure 3b) achieved an immunological and virological response during ART and had no treatment

interruptions. Levels of antibody reactive to JFH-1 and nAb₅₀ titres were low at the start of ART but increased during follow-up, correlating with control of HCV viral replication. A rebound in HIV viremia at week 278 for **P12** was associated with decreased CD4⁺ T-cell count, reduced JFH-1 antibody response and increased HCV viral load. For **P7** (Figure 3b), a decline in antibody responses after 368 weeks was associated with a rise in HCV viral load. With the exception of **P6**, all patients who displayed an inverse relationship between nAb₅₀ titres and HCV viral loads started ART with <100 CD4⁺ T-cells/ μ L and experienced significant immune recovery ($p < 0.05$ at 26, 104, 156 and 260 weeks).

A mixed pattern was demonstrated in **P2** who started ART with <50 CD4⁺ T-cell counts/ μ L and achieved an immunological and virological response to ART (Figure 3c). JFH-1 antibody levels and nAb₅₀ titres were low at week 4, but increased at 104 weeks with a concomitant rise in HCV viral load. Suppression of viral replication at week 152 paralleled a reduction in JFH-1 antibody and a HCV viral rebound at week 256 corresponded with an increase in JFH-1 antibody and nAb₅₀ titres. However, failure to maintain nAb responses after 474 weeks was linked to a dramatic rise in HCV RNA levels, so the antibody profile changed from pattern 1 to pattern 2.

3.7 Antibodies to JFH-1 declined on HCV therapy but antibodies to non-structural proteins increased with control of HCV replication

Four patients received anti-HCV therapy. **P1**, **P10** and **P14** were treated with IFN α /ribavirin for 48 weeks and achieved a sustained virological response, followed by lower JFH-1 antibody and nAb₅₀ titres (Figures 4a,b & c). HCV core antibody levels also declined in patients with sustained virological response (data not shown). *It is noteworthy that **P10** was able to control HCV replication despite having low nAb₅₀ titres compared to other patients. This may be due to*

the fact that this patient was infected with the genotype 2 strain of HCV which is the same as the JFH-1 strain used to assess neutralisation capacity. **P4** was treated with IFN α alone for 20 weeks without a virological response and retained high JFH-1 reactive antibody levels and nAb₅₀ titres (Figure 4d). As residual IFN α or ribavirin in the plasma may create false positives in the nAb assay, IgG was purified from **P1**, **P10** and **P14** plasmas collected before and after IFN α /ribavirin. All nAb₅₀ titres were comparable in purified IgG and the original samples (Supplementary Figure 4).

Antibodies to HCV non-structural proteins (NS3, NS4, NS5) rose with control of HCV replication on ART in several patients (notably **P2**, **P3**, **P5**, **P7**, **P10**, **P11**, **P15**; Figure 5 and **P9** and **P13** (not shown)).

4. Discussion

We present a comprehensive analysis of HCV-specific antibody responses in HIV/HCV co-infected patients followed for 11 years after initiating ART. We used antigens from cell cultures infected with the HCV genotype 2a virus, JFH-1, and commercially available recombinant proteins from HCV genotype 1. An advantage of using JFH-1 is the inclusion of envelope proteins (E1 or E2). We show that patterns of antibody responses differ with the antigen. Levels of JFH-1 and HCV core reactive antibodies were lower in untreated HIV/HCV patients than in HCV mono-infected patients. In contrast, levels of NS3 and NS5 antibody were high and NS4 antibodies were similar to HCV mono-infected patients.

Core is highly immunogenic, being recognized by 97% of patients with chronic HCV infection [19, 20]. In addition, levels of antibodies to core can be 10-fold higher than those reported for

E2, NS3, NS4 and NS5 [21]. Thus the lower JFH-1 antibody levels observed in HIV/HCV patients may reflect reduced levels of antibody to core, despite the high antibody levels to NS3 and NS5. Our findings differ from a report of weak reactivity to NS3, NS4 and NS5 antigens in HIV/HCV patients [22]. Increased NS3 and NS5 antibody levels could reflect the higher HCV viremia [23] expected in co-infected patients [24], though this was not evident here (Figure 5).

Total IgG was assessed to evaluate non-specific activation of B-cells. Levels were elevated before ART and did not normalise, suggesting continued B-cell activation by HIV and/or HCV disease. This fits with evidence that HIV/HCV co-infected patients treated for 1 year retained elevated IgG [25]. Similarly, HIV mono-infected patients but not HCV/HIV patients normalised their total IgG levels after 3 years on ART [9]. In contrast to several studies [26, 27], IgG levels in HCV mono-infected patients were not higher than healthy controls (data not shown). This may reflect their mild fibrosis (13/17 patients had fibrosis scores of 1 or 2), as serum IgG levels may correlate with the severity of hepatic fibrosis in HCV-infected patients [28].

The generation of nAb plays an important role in host immunity. In acutely HCV-infected patients, nAb contribute to the control of HCV replication and eventual eradication of the virus [29, 30]. However in chronically-infected humans and chimpanzees [31] and in HIV/HCV patients with controlled HIV viremia [13], nAb₅₀ titres did not correlate with HCV RNA levels. Here, genotype cross-reactive nAb₅₀ titres were lower in untreated co-infected patients compared to HCV mono-infected patients, with undetectable or low titres of nAb in nine co-infected individuals. These patients may have antibodies that do not cross-neutralise with JFH-1.

Baseline JFH-1 antibody levels correlated with CD4⁺ T-cell counts in HIV/HCV patients. Accordingly, antibodies to HCV core, NS3, NS4 and NS5 antigens were lowest in untreated HIV/HCV patients with CD4⁺ T-cell counts below 200 cells/ μ L [32]. Here, antibody responses increased on ART, but only nAb₅₀ titres reached levels seen in HCV mono-infected patients. We also demonstrated a significant increase in JFH-1 antibody levels and nAb₅₀ titres in longitudinal samples from Indonesian HIV/HCV patients after 1 year of ART [18]. In a cross-sectional study of HIV/HCV patients who had controlled HIV viremia during ART, nAb₅₀ titres were significantly lower than HCV mono-infected patients [13], but the duration of treatment was not specified. Here, nAb₅₀ titres were significantly lower than HCV mono-infected patients during the first 4 years but normalised with longer periods of ART (Figure 2).

Recovery of antibody responses on ART was also evident with CMV, and may reflect restored T-cell help for B-cell responses as it paralleled improved HCV-specific CD4⁺ and CD8⁺ T-cell responses [3, 4]. This trend over time was evident in our study, but increases in JFH-1 antibody responses on ART were independent of CD4⁺ T-cell counts at recruitment. In contrast, cell-mediated T-cell responses to HCV antigens assessed by lymphoproliferation and interferon- γ ELISPOT were associated with nadir CD4⁺ T-cells counts [33, 34]. Interestingly, patients who started ART with low CD4⁺ T-cell counts developed nAb responses more rapidly than patients with high CD4⁺ T-cell counts and displayed an inverse correlation between nAb₅₀ titres and HCV viral loads. Whilst this appears counterintuitive, virus-specific protective antibody responses were improved by partial CD4⁺ T-cell depletion or peptide tolerisation of virus-specific CD4⁺ T-cells in murine lymphocytic choriomeningitis virus infection [35, 36]. The authors postulated that T-cell dependent polyclonal activation of B-cells reduced activation of virus-specific B-cells and the formation of high affinity nAb. In several patients, nAb responses

failed to control HCV viral replication. In these individuals, virus-specific CD8⁺ T-cell responses may have a role in controlling viremia [37].

Liver enzyme levels increase in some HIV/HCV co-infected patients beginning ART [5-8]. This has been attributed to particular antiretroviral drugs or restoration of HCV-specific immune responses. Patients with elevated serum ALT levels can display high levels of HCV core antibody [8] and/or HCV antibodies assessed by diagnostic kits [7]. In the current study, antibody responses increased irrespective of changes in serum ALT during ART. Furthermore, median levels of antibody reactive with JFH-1 were similar in individuals with or without elevated ALT. However, we did not assess antibody responses between baseline and 26 weeks where most differences occur [7]. Similarly, JFH-1 reactive antibody levels were significantly raised in Indonesian patients with liver enzyme elevations at baseline and during the first 12 weeks of ART but not at 24 and 48 weeks [18]. Further work is required to elucidate if HCV-specific antibodies play a direct role in hepatotoxicity.

Suppression of HCV replication by anti-HCV therapy was associated with reduced nAb₅₀ titres, and antibodies to JFH-1, core and non-structural antigens (Figure 4). This suggests that the presence of antigen may boost production of antibodies. This association has been reported for several viruses including HCV [38] and HIV [39] and is the general basis for immunisation. For HIV/HCV patients treated with pegylated IFN α and ribavirin, sustained virological responses paralleled reductions in antibodies to HCV core and NS4 [40]. Accordingly, HCV mono-infected patients who resolved HCV infection without therapy had lower levels of antibodies to NS3 and NS5 than individuals with persistent viremia [23]. Furthermore, in patients with chronic HCV infection and a sustained virological response following IFN α therapy, antibody to NS3, NS4

and NS5 declined for up to 10-years follow-up [41, 42]. A decline in antibody levels with HCV therapy seen here contradicts two studies in which clearance of HCV RNA following pegylated IFN α and ribavirin therapy did not change nAb₅₀ titres to JFH-1 [43] or HCV pseudoparticles [44] in HCV mono-infected patients. This could reflect the short follow-up periods (<12 months). We suggest that antibody responses to JFH-1 and/or recombinant antigens may help in assessing outcome of HCV therapy. It is important to note that antibodies to NS3, NS4 and NS5 rose following clearance of HCV RNA on ART (Figure 5), presumably by immunological mechanisms.

Limitations of this study include the small sample size and the heterogeneity of treatment regimes. Second, the use of plasma or serum in our neutralisation assay may be confounded by the presence of soluble factors that can affect virus infectivity in cell culture. However, we demonstrated strong positive correlations for nAb₅₀ titres when plasma or purified IgG from 51 HCV mono-infected patients was used for the assay ($r=0.85$, $p<0.0001$, unpublished data). Third, the neutralisation assay utilised a HCV genotype 2a virus strain, whilst most patients were infected with genotype 1 or 3. This may result in the underestimation of nAb₅₀ titres. However, in 99 chronically HCV mono-infected patients [genotype 1 (n=47); genotype 2 (n=19), genotype 3 (n=31); genotype 4 (n=2)], nAb₅₀ titres were independent of HCV genotype (unpublished data). Nevertheless, there is a need for additional work using pseudoparticles encoding autologous E1-E2 sequences to confirm the undetectable or low nAb responses reported in several HIV/HCV patients. Fourth, we cannot conclude that a reduction in HCV viral loads was a direct consequence of increased nAb₅₀ titres, as there may be concomitant generation of HCV-specific CD4⁺ and CD8⁺ T-cell responses. [It is well established that the presence of vigorous and multi-specific HCV-specific CD8 T-cell responses are associated with spontaneous viral](#)

clearance in acutely infected humans and chimpanzees [45, 46] and sustained virological responses in chronically infected patients receiving IFN α and ribavirin combination therapy [47, 48]. Further studies monitoring both humoral and cell-mediated immune responses at regular intervals in individual patients are warranted. However, considering these limitations, these longitudinal results builds a picture of how HCV antibody responses change during long-term ART in patients co-infected with HIV and HCV.

In summary, antibody responses measured using the JFH-1 virus increase with long-term ART irrespective of baseline CD4⁺ T-cell counts. nAb responses correlate with control of HCV replication in some patients.

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Figure 1. Levels of circulating total IgG (a), CMV antibody (b) and antibodies to JFH-1 (c), HCV core (d), NS3 (e), NS4 (f), NS5 (g) and HCV nAb₅₀ titres (h) in HIV/HCV co-infected patients, HCV mono-infected patients and healthy controls. JFH-1 antibody levels were determined by subtracting the value obtained with uninfected cells from the value for JFH-1 lysate and averaged over several sample dilutions. The 50% neutralising antibody (nAb₅₀) titre was the plasma dilution required to inhibit JFH-1 infection by 50%. Lines represent median values. *p*-values were determined using Mann Whitney T-Tests.

Figure 2. Levels of total IgG (a), CMV antibody (b), JFH-1 antibody (c) and nAb₅₀ titres (d) in longitudinal samples from 17 HIV/HCV co-infected patients initiating ART. Longitudinal data were grouped to the nearest timepoint. The black lines represent the median values for HIV/HCV co-infected patients (a-d). The grey dotted lines represent the median values for healthy controls (a and b) or HCV mono-infected patients (c and d) and the shaded areas represent the 25th and 75th percentile. JFH-1 antibody levels were determined by subtracting the value obtained with uninfected cells from the value for JFH-1 lysate and averaged over several sample dilutions. The 50% neutralising antibody (nAb₅₀) titre was the plasma dilution required to inhibit JFH-1 infection by 50%.

Figure 3. Immunological, virological and biochemical details (top panel) and JFH-1 specific antibody responses (bottom panel) in longitudinal samples from HIV/HCV co-infected patients on ART. For the top panel, the *darker grey shaded areas* represent the HCV viral loads, the *open circle line graph* show the serum ALT levels and the *closed diamond line graph* represent the CD4⁺ T-cell counts. For the bottom panel, the *open circle line graph* represent nAb₅₀ titres and the *closed circle line graph* show JFH-1-reactive antibody levels. Time on ART

is represented by grey shaded areas. HIV viral loads were considered detectable (+) if above 3.0 log₁₀ copies/mL. JFH-1 antibody levels were as described in Figure 3. The 50% neutralising antibody (nAb₅₀) titre was the plasma dilution required to inhibit JFH-1 infection by 50%.

Figure 4. Immunological, virological and biochemical details (left panel) and JFH-1 specific antibody responses (right panel) in longitudinal samples from HIV/HCV co-infected patients who achieved a sustained virological response (a-c) or did not respond following IFN α -based therapy (d). For the left panel, the *darker grey shaded areas* represent the HCV viral loads, the *open circle line graph* show the serum ALT levels and the *closed diamond line graph* represent the CD4⁺ T-cell counts. For the right panel, the *open circle line graph* represent nAb₅₀ titres and the *closed circle line graph* show the JFH-1-reactive antibody levels. Time on ART or IFN α -based therapy are represented by grey shaded areas or hatched bars respectively. HIV viral loads were considered detectable (+) if above 3.0 log₁₀ copies/mL. JFH-1 antibody levels were as described in Figure 3. The 50% neutralising antibody (nAb₅₀) titre was the plasma dilution required to inhibit JFH-1 infection by 50%.

Figure 5. Antibody levels to recombinant HCV antigens in longitudinal samples from 7 HIV/HCV co-infected patients on ART. The *closed circle line graph* represent antibody levels to HCV core, the *open circle line graph* show antibody levels to HCV NS3, the *closed diamond line graph* represent antibody levels to HCV NS4 and the *open diamond line graph* show antibody levels to HCV NS5. Time on ART or IFN α -based therapy are represented by light grey shaded areas or hatched bars respectively. HIV viral loads were considered detectable (+) if above 3.0 log₁₀ copies/mL. The dark grey shaded areas indicate HCV viral loads. JFH-1 antibody

levels were as described in Figure 3. The 50% neutralising antibody (nAb₅₀) titre was the plasma dilution required to inhibit JFH-1 infection by 50%.

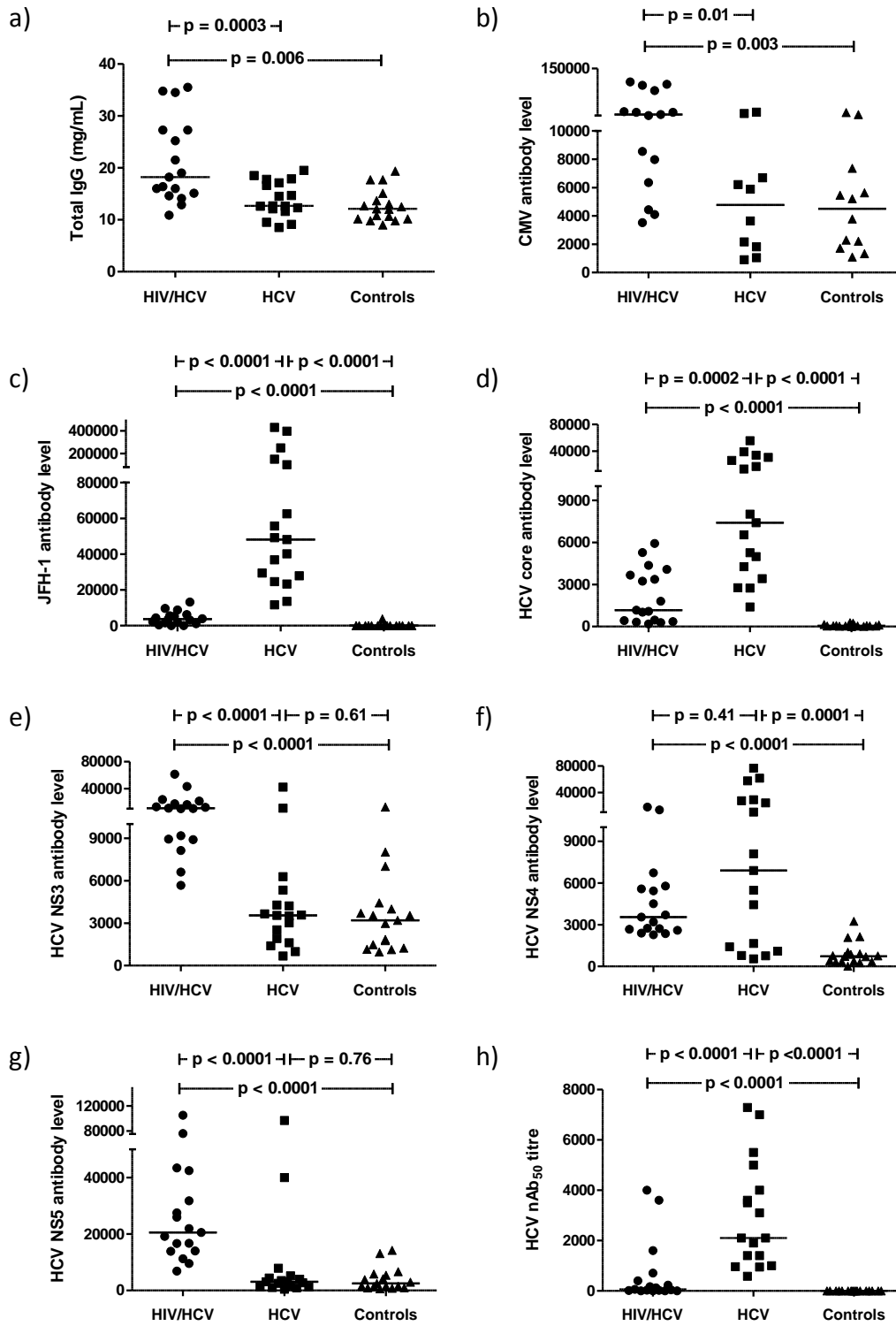


Figure 1

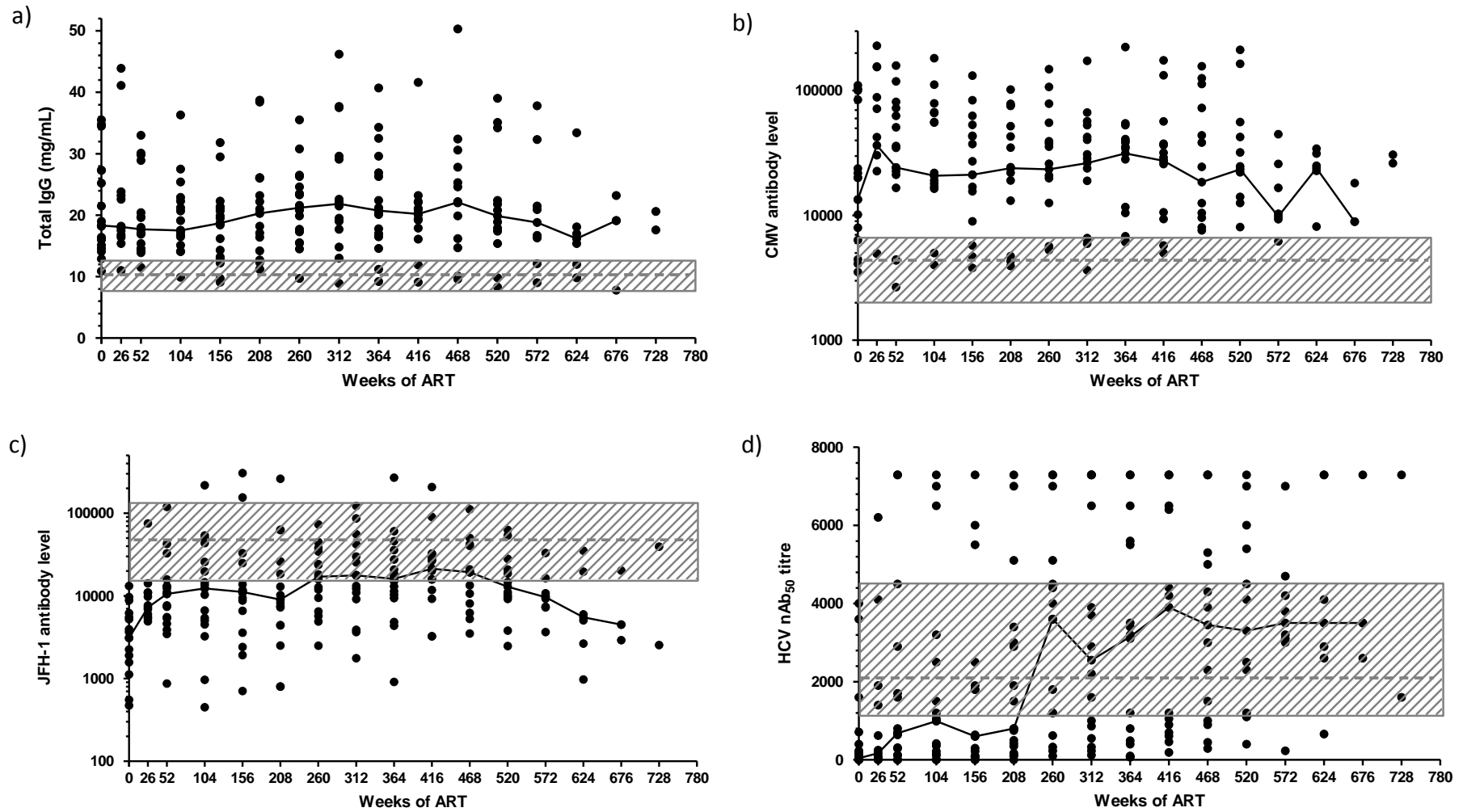


Figure 2

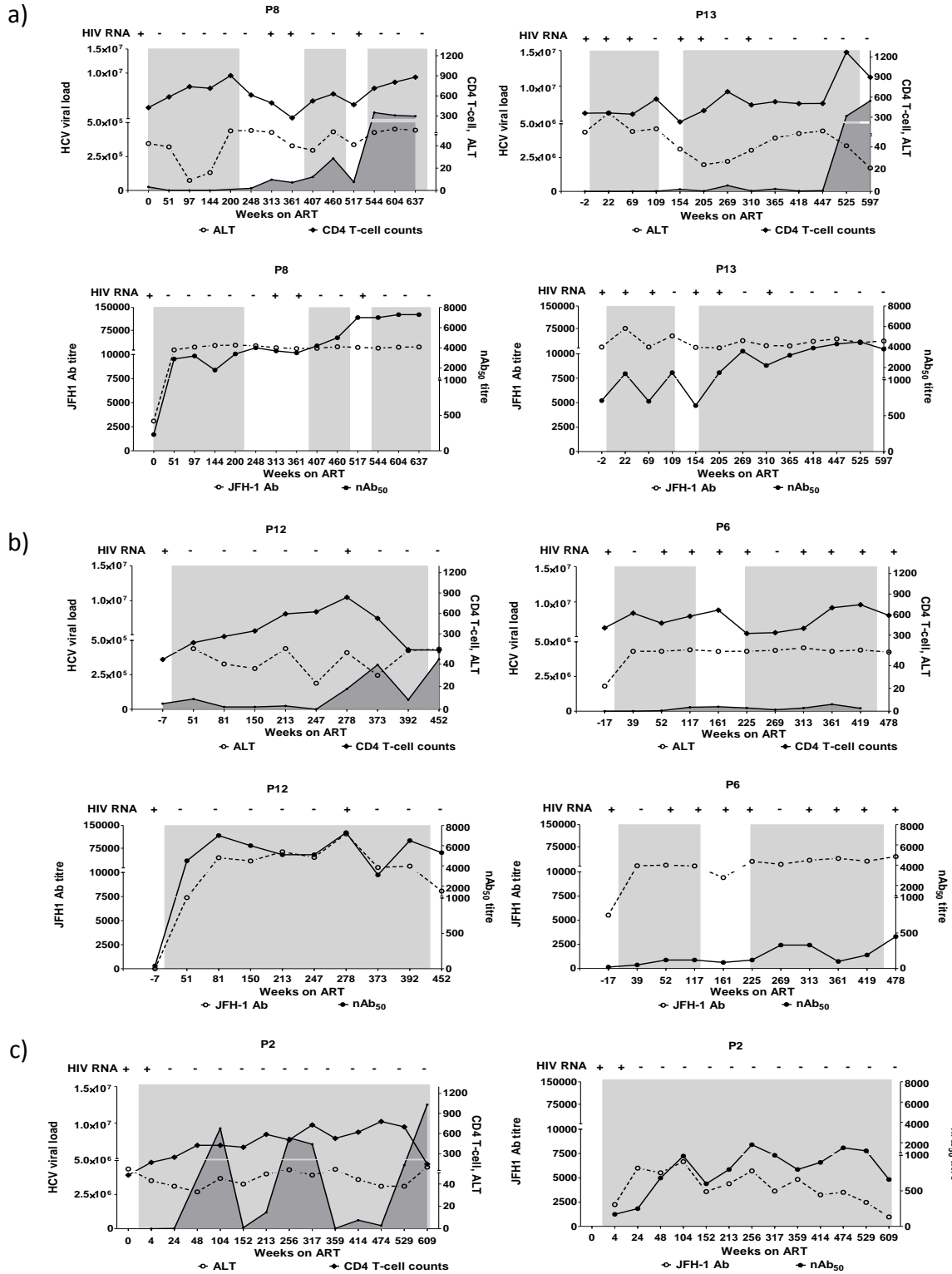


Figure 3

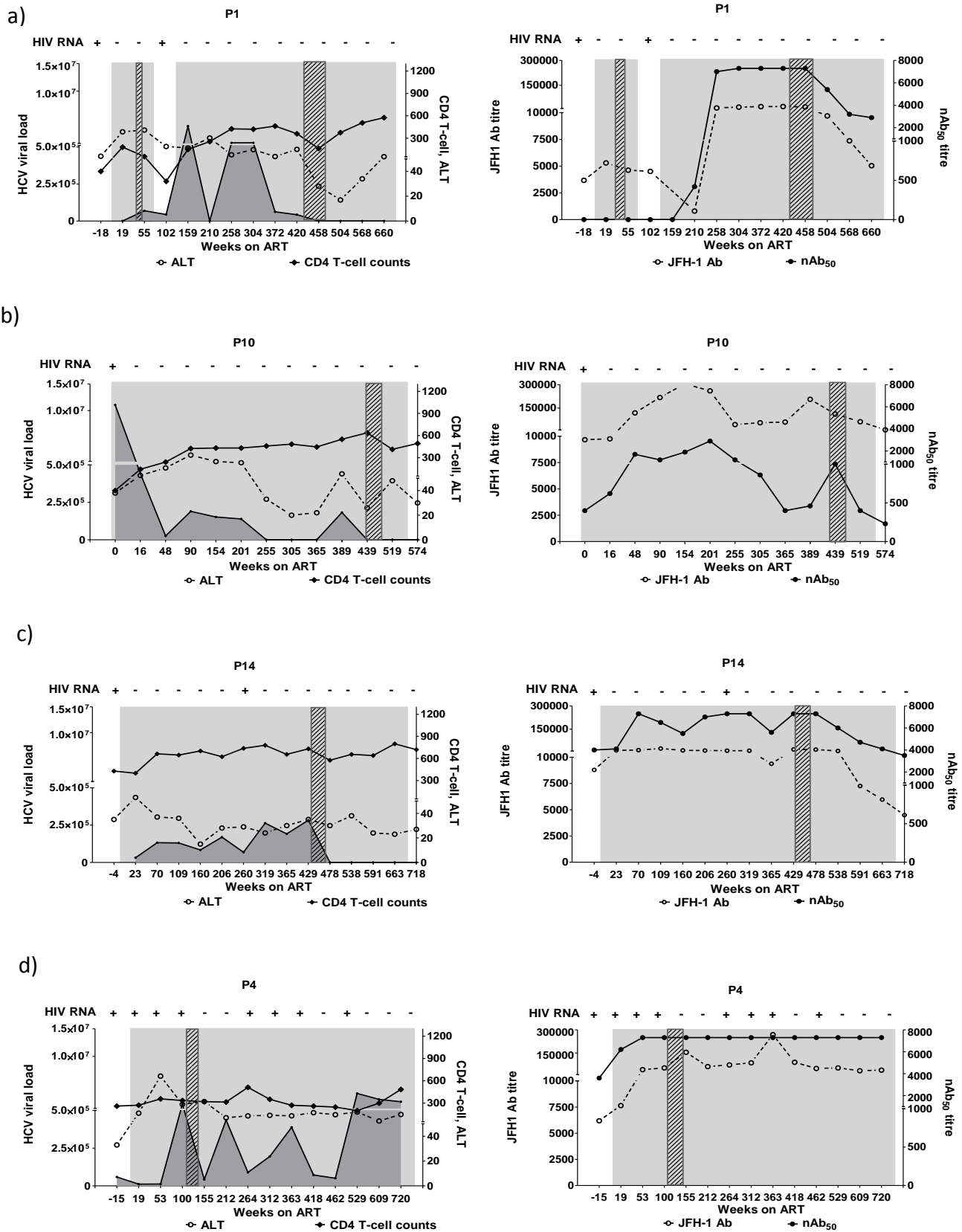


Figure 4

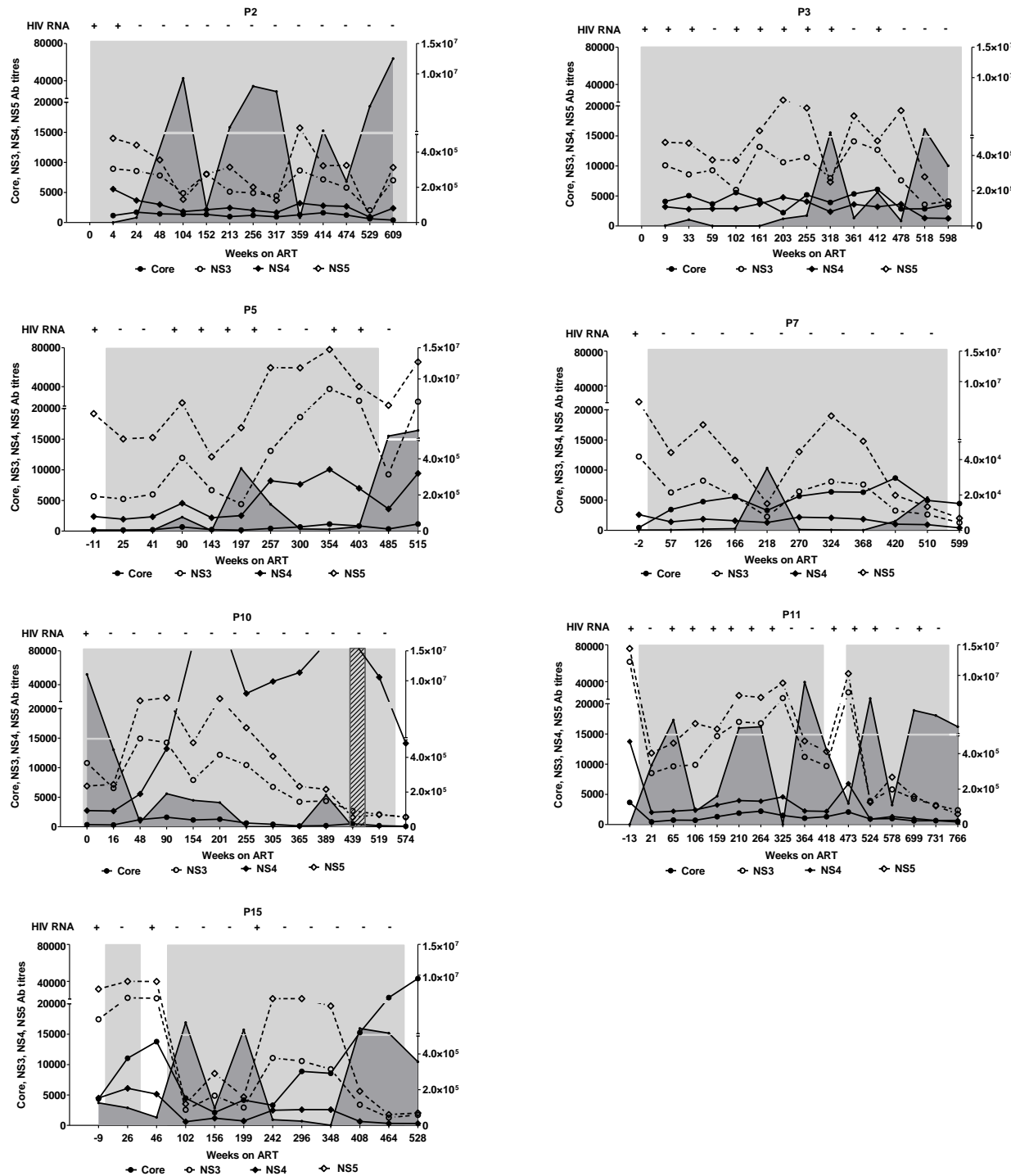


Figure 5

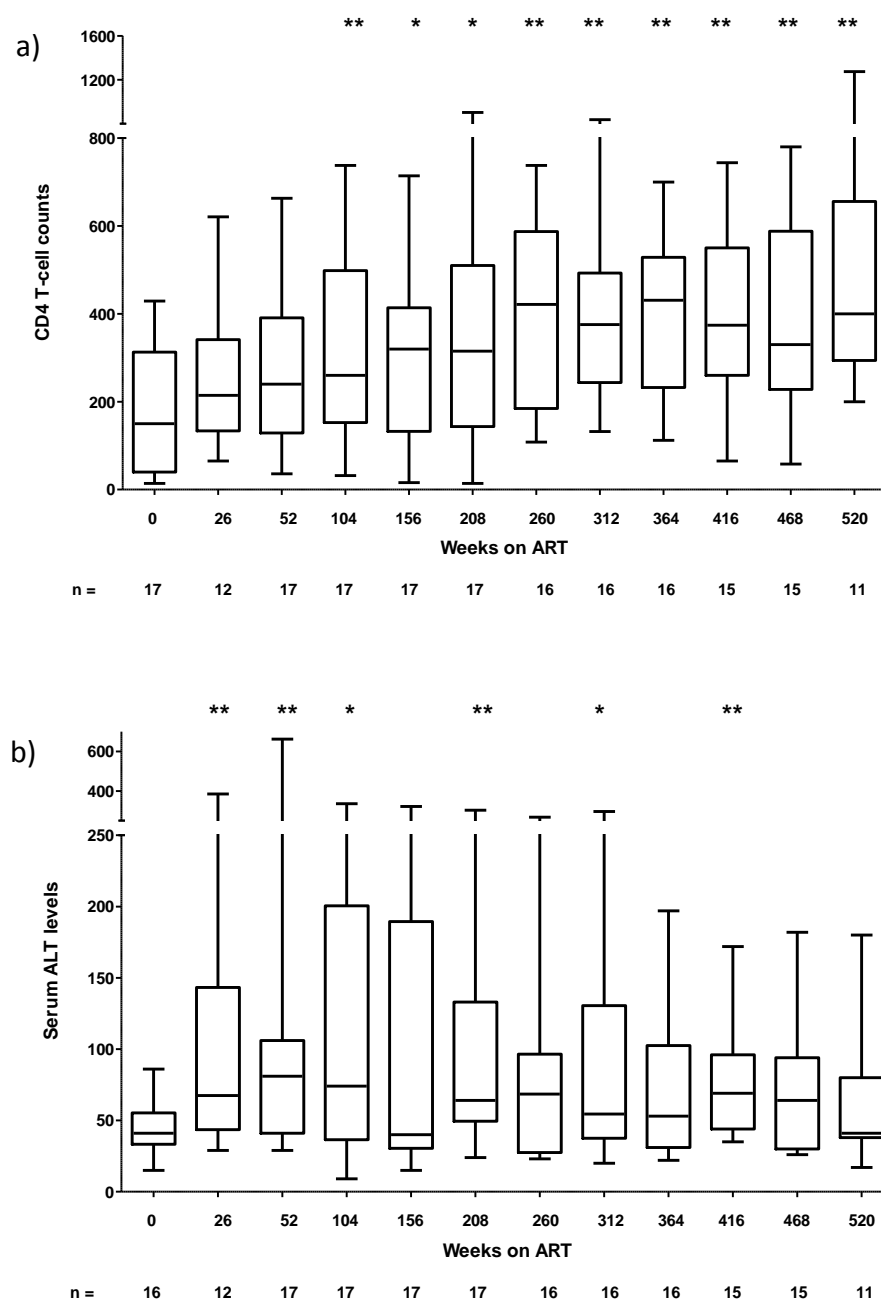
Table 1. Characteristics of HIV/HCV co-infected patients

Patient ID	Age*	Sex	Race	HCV genotype	Follow-up (weeks)		CD4 ⁺ T-cell count*	CD8 ⁺ T-cell count*	Serum ALT*	HIV viral load* [#]	HCV viral load [^]	HCV therapy
P1	35	M	W	1	-18	660	40	552	58	5.45	833 ¹	NR ^a , SVR ^b
P2	43	M	W	1	0	609	48	220	71	5.88	357 ²	NT
P3	33	M	W	1	0	598	112	322	51	4.75	2022 ³	NT
P4	43	M	W	1	-15	720	260	793	33	3.68	59238	NR ^a
P5	28	M	AS	1	-11	515	150	825	86	3.01	1903	NT
P6	30	M	W	1	-17	478	405	795	22	4.50	< 200	NT
P7	37	M	W	1	-2	599	30	750	15	5.48	< 200	NT
P8	32	F	W	1	0	637	425	952	42	4.16	26051	NT
P9	37	M	AS	3	0	465	266	1273	28	4.02	< 200	NR ^a
P10	34	M	AS	2	0	574	40	490	38	5.33	11062454	SVR ^b
P11	37	M	W	3	-13	766	20	168	40	5.58	< 200	NT
P12	36	M	W	ND	-7	452	44	869	ND	6.60	40920	NT
P13	40	M	W	1	-2	597	360	774	76	5.02	< 200	NT
P14	34	F	W	1	-4	718	429	341	35	4.36	31998 ⁴	SVR ^b
P15	32	M	W	1	-9	528	65	910	34	4.62	126327	NT
P16	30	M	AB	1	-2	393	210	798	56	4.88	1546	NT
P17	29	F	W	ND	-36	186	64	1168	321	5.55	< 200	NT

W: White, AS: Asian, AB: Aboriginal, ND: Not determined, SVR: Sustained Virological Response, NR: Non-responders, NT: Not treated

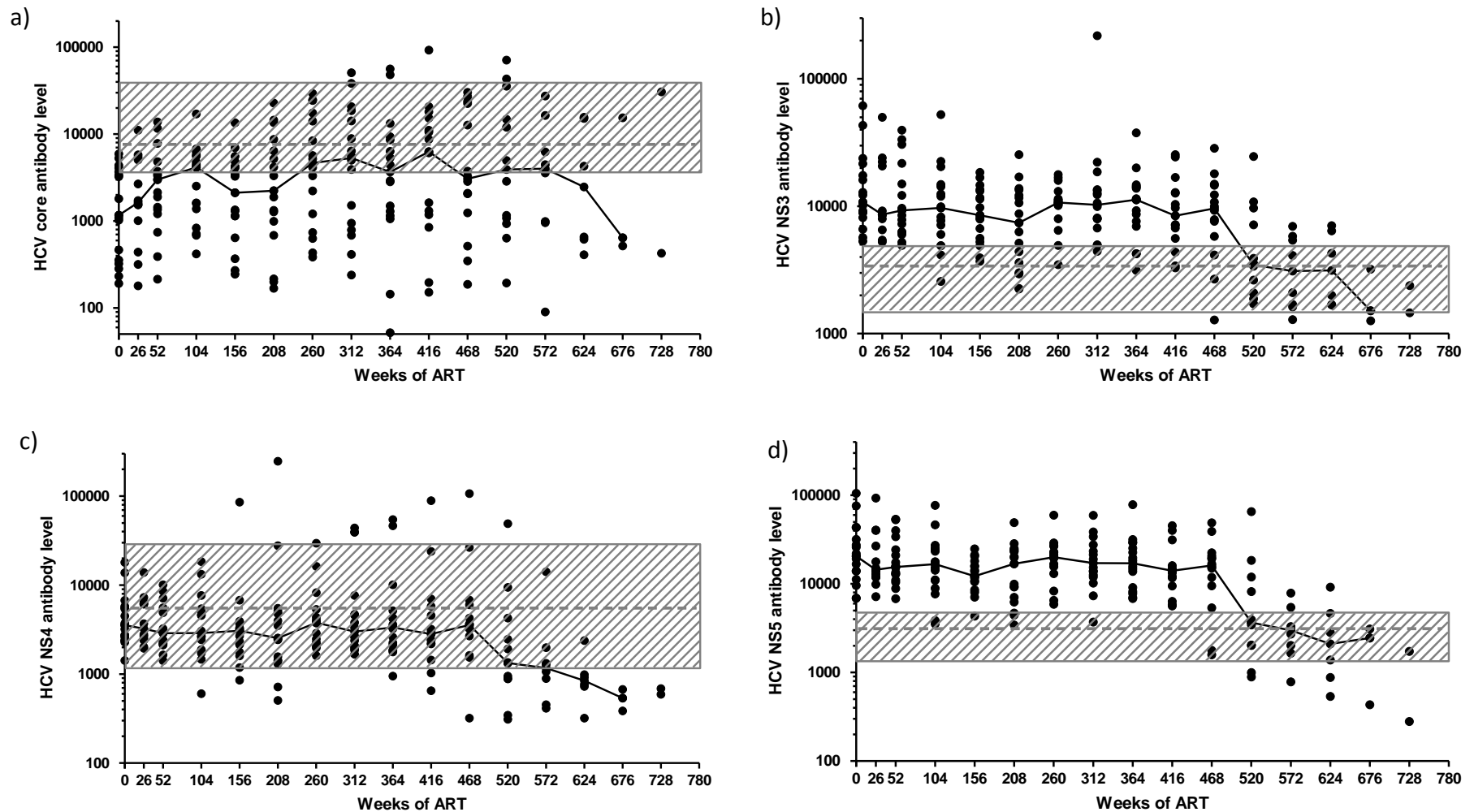
Reference ranges for healthy individuals: CD4⁺ T-cell count (457 – 1498) and CD8⁺ T-cell count (205 – 1013)

* At study entry, [#] log₁₀ copies/mL, [^] copies/mL, ^a IFN α monotherapy, ^b IFN α and ribavirin combination therapy, ¹ 19 weeks follow-up, ² 4 weeks follow-up, ³ 9 weeks follow-up, ⁴ 23 weeks follow-up

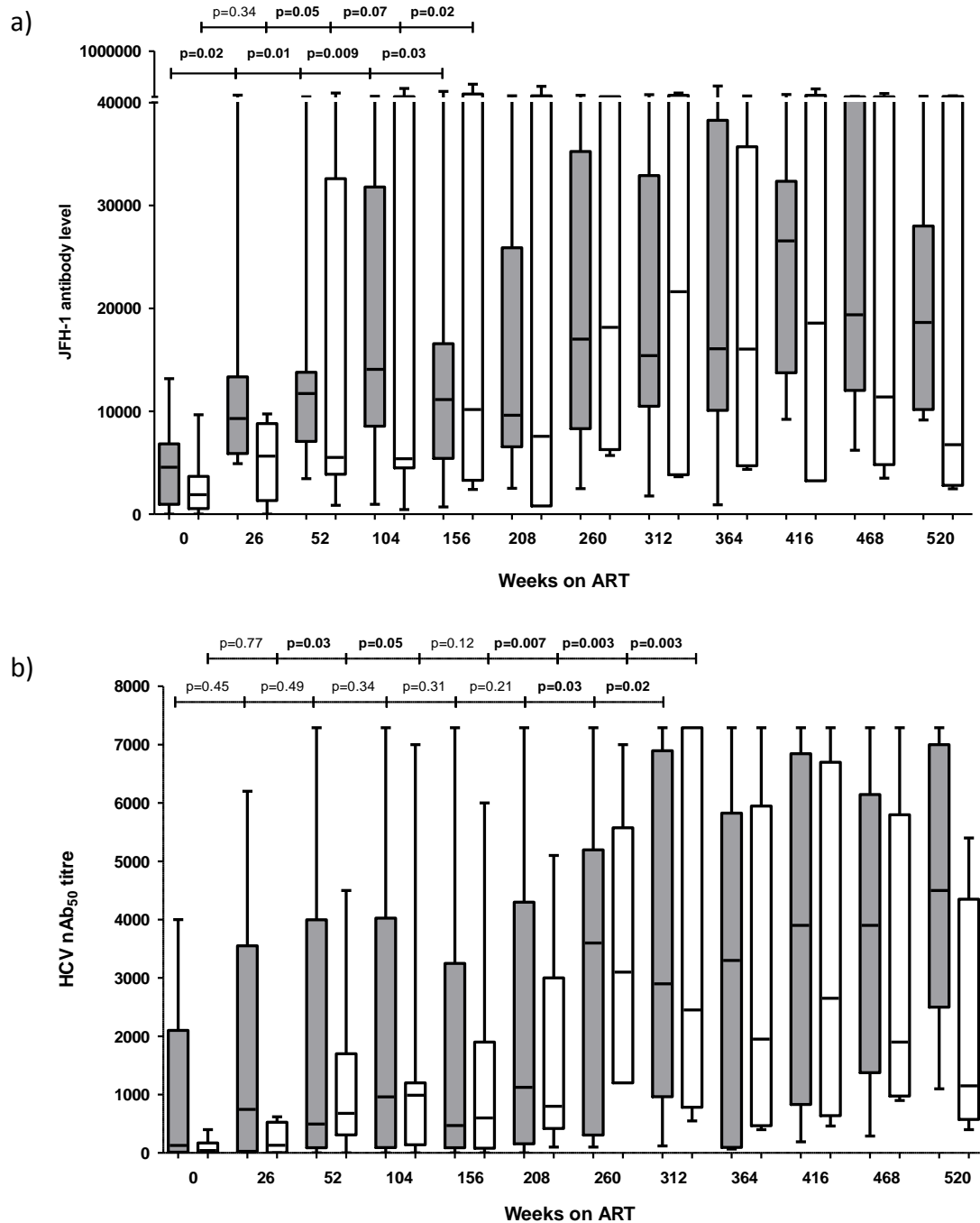


Supplementary Figure 1. CD4⁺ T-cells counts (a) and serum ALT levels (b) in longitudinal samples from HIV/HCV co-infected patients on ART. Longitudinal data were grouped to the nearest time-point. Data are presented as box and whisker plots where the lower and upper limits of the central box represent the 25th and 75th percentile. The middle line represents the median.

** p -value <0.05, * p -value 0.05-0.10 compared to baseline

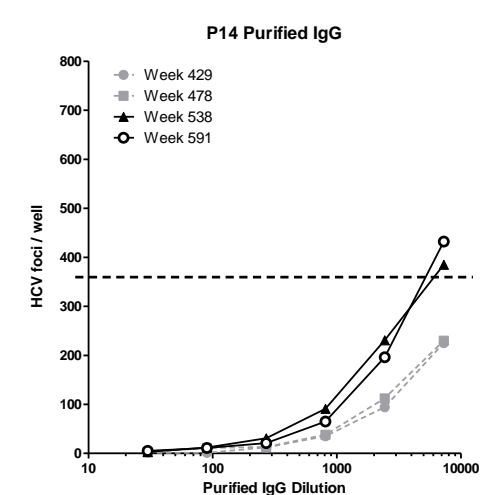
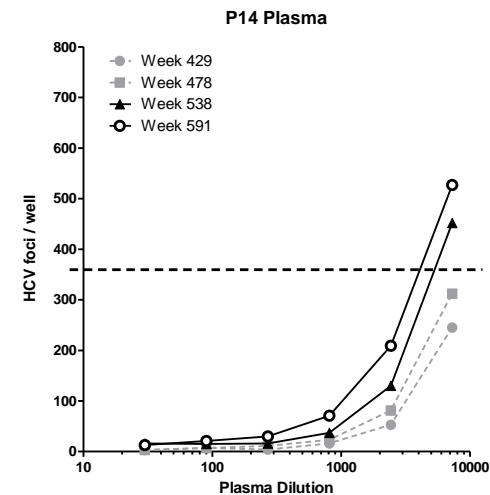
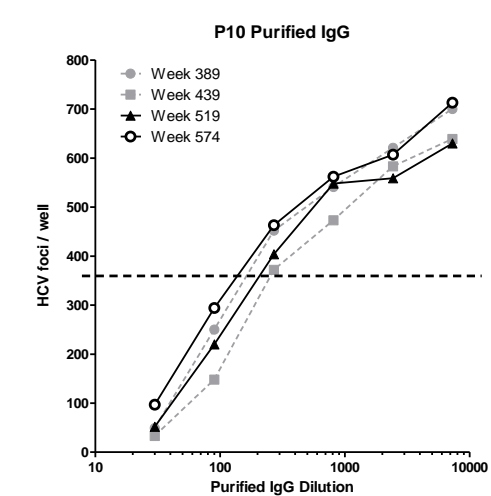
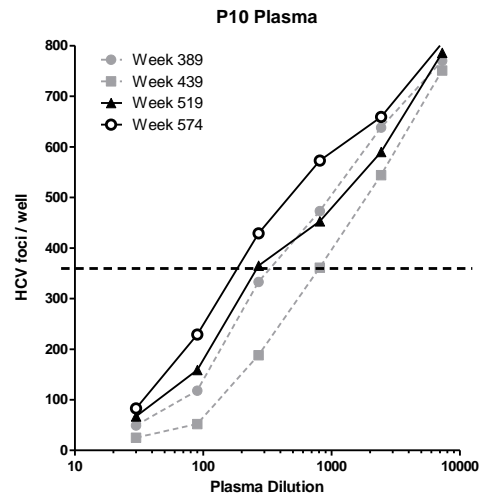
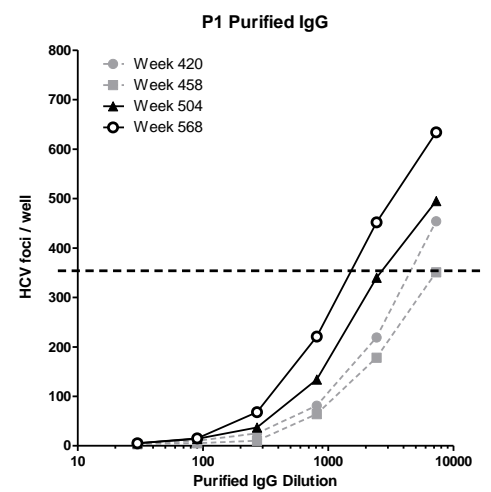
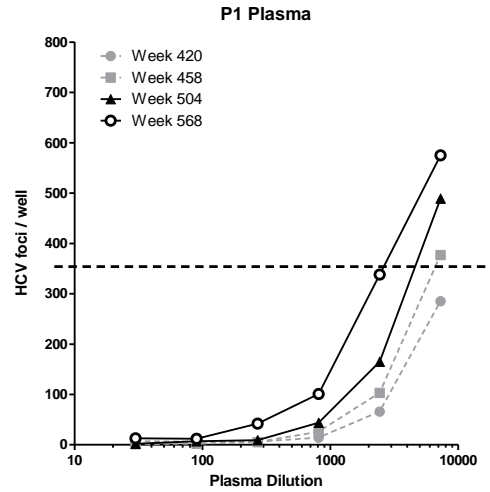


Supplementary Figure 2. Antibody levels to HCV core (a), NS3 (b), NS4 (c) and NS5 (d) in longitudinal samples for 17 HIV/HCV co-infected patients on ART. Longitudinal data were grouped to the nearest time-point. The black lines represent the median values for HIV/HCV co-infected patients (a-d). The grey dotted lines represent the median values for HCV mono-infected patients and the shaded areas represent the 25th and 75th percentile.



Supplementary Figure 3. Levels of JFH-1-reactive antibody (a) and nAb₅₀ titres (b) in longitudinal samples from HIV/HCV co-infected patients stratified as having CD4⁺ T-cell counts of >50 (grey bars) (n=10) or <50 (white bars) (n=7) cells/ μ L at study entry. Longitudinal data were grouped to the nearest time-point. Data are presented as box and whisker plots where the lower and

upper limits of the central box represent the 25th and 75th percentile. The middle line represents the median. JFH-1 antibody levels were determined by subtracting the value obtained with uninfected cells from the value for JFH-1 lysate and averaged over several sample dilutions. The 50% neutralising antibody (nAb₅₀) titre was the plasma dilution required to inhibit JFH-1 infection by 50%.



Supplementary Figure 4. Neutralisation curves for plasma and purified IgG from 3 HIV/HCV co-infected patients (P1, P10 and P14). Capacity for neutralisation was compared between plasma (left) and IgG purified using Protein G (right). Two samples were collected during IFN α /ribavirin therapy (grey dotted lines) and two samples after treatment (black solid lines).

Supplementary Table 1. Clinical characteristics

	HIV/HCV (n=17)	HCV (n=17)	Controls (n=17)	<i>p</i>-value*
Age (years)	34 (28 – 43)	39 (29 – 49)	57 (30 – 74)	0.003
Sex (M / F)	14 / 3	14 / 3	15 / 2	
ALT (U/mL)	41 (15 – 321)	69 (21 – 314)	ND	0.013
CD4 ⁺ T-cell count	150 (20 – 429)	938 (637 – 2142)	ND	<0.0001
CD8 ⁺ T-cell count	793 (168 – 1273)	597 (273 – 1428)	ND	0.552
HCV genotype				
1 / 2 / 3 / ND	11 / 1 / 3 / 2	11 / 1 / 3 / 2		

Results are present as median (range). *p*-values were determined using Mann Whitney T Test. ND, Not determined

*HIV/HCV *vs* HCV